Form PTO 1390 (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER P32085
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/743209
INTERNATIONAL APPLICATION NO. PCT/GB99/02301	INTERNATIONAL FILING DATE 15 July 1999	PRIORITY DATE CLAIMED 17 July 1998
TITLE OF INVENTION PROCESS FOR PREPARING CLAVAM DERIVATIVES BY USING POLYPEPTIDES HAVING BETA-LACTAM SYNTHETASE ACTIVITY		
APPLICANT(S) FOR DO/EO/US Barry BARTON, Heather Jane MCNAUGHTON, Christopher Joseph SCHOFIELD, and Jan Edward THIRKETTLE		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1 [x] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [x] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [x] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [x] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [x] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).

6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S. C. 371(c)(3)).

9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [x] An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. [x] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
    [ ] Please amend the specification by inserting before the first line the sentence: This is a 371 of International Application PCT/GB99/02301, filed 15 July 1999, which claims benefit from the following Provisional Application: GB 9815666.4 filed 17 July 1998.

14. [ ] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [x] Other items or information: Sequence listing, Statement to Support, diskette

US APPLICATION NO/(if known, see 37 CFR 1.5) 09/744,109	INTERNATIONAL APPLICATION NO. PCT/GB99/02301	ATTORNEYS DOCKET NO. P32085

17. [X] The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 C.F.R. 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO **$860.00**		
International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) **$690.00**		
No International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) **$710.00**		
Neither International Preliminary Examination Fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....**$1,000.00**		
International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	**$0.00**	

Claims	Number Filed	Number Extra	Rate		
Total claims	**14 - 20 =**	**0**	**0 x $18.00**	**$0.00**	
Independent claims	**2 - 3 =**	**0**	**0 x $80.00**	**$0.00**	
Multiple dependent claims (if applicable)			**+ $270.00**	**$270.00**	
			TOTAL OF ABOVE CALCULATIONS =	**$270.00**	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				**$**	
			SUBTOTAL =	**$1130.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)) +				**$**	
			TOTAL NATIONAL FEE =	**$1130.00**	
			Amount to be refunded	**$**	
			charged	**$**	

a. ☐ A check in the amount of $____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 19-2570 in the amount of **$1130.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-2570 . A duplicate copy of this sheet is enclosed.

d. ☒ General Authorization to charge any and all fees under 37 CFR 1.16 or 1.17, including petitions for extension of time relating to this application (37 CFR 1.136 (a)(3)).

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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"EXPRESS MAIL CERTIFICATE"
"EXPRESS MAIL" MAILING LABEL NUMBER EL421190155US
DATE OF DEPOSIT: 08 January 2001

Attorney Docket No. P32085

IN THE **UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant: Barton, et al. 08 January 2001

International App. No.: PCT/GB99/02301 Group Art Unit No.: Unknown

International Filing Date: 15 July 1999 Examiner: Unknown

For: NOVEL COMPOSITION

Assistant Commissioner of Patents
Box: PCT
Washington, D.C. 20231

PRELIMINARY AMENDMENT

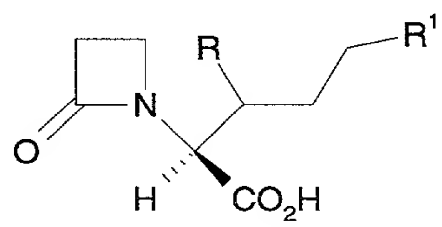
Preliminary to the examination of this application, applicants respectfully request amendment of the above-identified application as follows:

IN THE CLAIMS:

Please cancel claims 1-14.

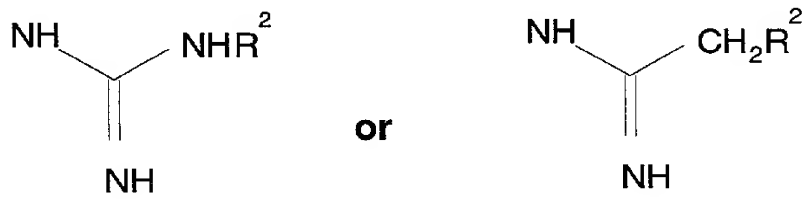
Please add new claims 15- 28.

15. A process for preparing compounds of formula (I)



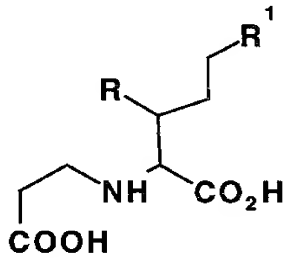
(I)

wherein R is H or OH and R^1 is



and where R^2 = H or C_{1-6} alkyl

by contacting a compound of formula (II)

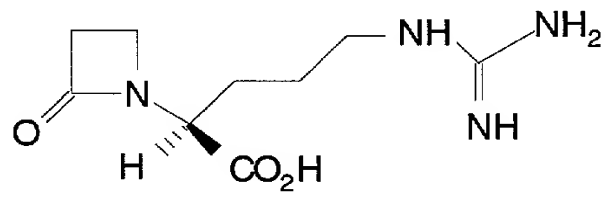


(II)

where the variables are as defined in formula (I)

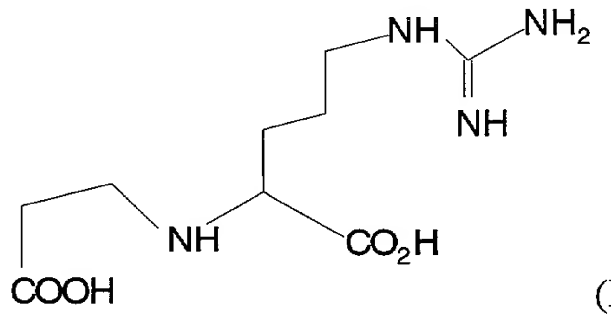
with a polypeptide having at least 95% identity to the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2, and having β-lactam synthetase activity.

16. A process for preparing a compound of formula (IV)



(IV)

by contacting N^2-(2-carboxyethyl)-(S)-arginine; formula (III)



(III)

with a polypeptide having at least 95% identity to the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2, and having β-lactam synthetase activity.

17. A process according to claim 15 or 16 wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:2.

18. A process according to claim 15 or 16 wherein the polypeptide has the amino acid sequence of SEQ ID NO:2.

19. A process according to claim 15 or 16 wherein the polypeptide having β-lactam synthetase activity is obtainable from *Streptomyces* species.

20. A process according to claim 19 wherein the *Streptomyces* species is *Streptomyces clavuligerus*.

21. A recombinant vector comprising a polynucleotide capable of producing the polypeptide defined in claim 15 when said vector is present in a compatable host.

22. A recombinant vector according to claim 21 comprising a polynucleotide selected from the group:

a) a polynucleotide encoding a polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2;

b) a polynucleotide comprising the polynucleotide sequence of SEQ ID NO:1; or

c) a polynucleotide having the polynucleotide sequence of SEQ ID NO:1.

23. A process according to claim 15 or 16 wherein the polypeptide is expressed from a recombinant vector comprising a polynucleotide capable of producing a polypeptide having at least 95% identity to the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2, and having β-lactam synthetase activity, when said vector is present in a compatible host.

24. A host microorganism containing a recombinant vector of claims 21 or 22.

25. A host microorganism according to claim 24 which is selected from *Streptomyces*, or *E.coli*.

26. A process for preparing an enzyme having β-lactam synthetase activity which comprises the steps:

a) culturing *Streptomyces clavuligerus*,

b) harvesting and lysing the mycelium, and

c) isolating a polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2, and having β-lactam synthetase activity.

27. A process for preparing an enzyme having β-lactam synthetase activity which comprises the steps:

a) culturing a host microorganism transformed with a recombinant vector according to claims 21 or 22, and

b) isolating the polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2, and having β-lactam synthetase activity.

28. A process for preparing clavulanic acid comprising preparing a compound of formula (IV) in accordance with claim 16 or any claim dependent thereon, and then converting the compound of formula (IV) to clavulanic acid by treatment with an enzyme system derived from *Streptomyces clavuligerus*.

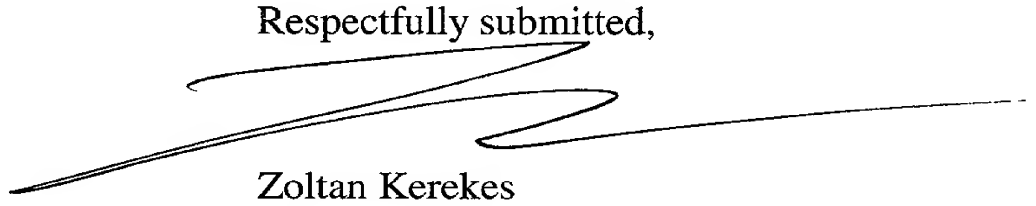
REMARKS

The above-identified application is being entered into the National Phase from PCT application no. PCT/GB99/02301.

Applicants have cancelled claims 1-14 and added new claims 15-28 to put the claims in conformity with U.S. practice.

No new matter has been introduced.

Respectfully submitted,



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DATE OF DEPOSIT: 08 January 2001

Attorney Docket No.: P32085

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Barton, et al. January 08, 2001

Serial No.: PCT/GB99/02301 Group Art Unit No.: Unknown

Filed: 15 July 1999 Examiner: Unknown

For: "Process for Preparing Clavam Derivatives by Using Polypeptides Having Beta-Lactam Synthetase Activity

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 CFR §§ 1.821 THROUGH 1.825

BOX SEQUENCE
Assistant Commissioner for Patents
Washington, D.C. 20231

(X) I hereby state that the contents of the paper and computer readable copies of the Sequence Listing, submitted in accordance with **37 CFR §1.821(c)** and **(e)**, respectively, are the same.

( ) I hereby state that the submission filed in accordance with **37 CFR §1.821 (g)** does not include new matter.

( ) I hereby state that the submission filed in accordance with **37 CFR §1.821 (h)** does not include new matter or go beyond the disclosure in the international application as filed.

( ) I hereby state that the amendments, made in accordance with **37 CFR §1.825 (a)**, included in the substitute sheet(s) of the Sequence Listing are supported in the application, as filed, at pages _____. I hereby state that the substitute sheet(s) of the Sequence Listing does (do) not include new matter.

( ) I hereby state that the substitute copy of the computer readable form, submitted in accordance with **37 CFR §1.825(b)**, is the same as the amended Sequence Listing.

PROCESS FOR PREPARING CLAVAM DERIVATIVES BY USING POLYPEPTIDES HAVING BETA-LACTAM SYNTHETASE ACTIVITY

The present invention relates to a new process for the synthesis of clavam compounds, in particular clavulanic acid. In one aspect the invention relates to β-lactam synthetase polypeptides and β-lactam synthetase polynucleotides, recombinant materials thereof and methods for their production. In another aspect, the invention relates to methods for using such polypeptides and polynucleotides.

A common feature of all penicillins and cephalosporins is the presence of a β-lactam ring which is vital to their antibiotic activity. β-lactam compounds are susceptible to degradation by β-lactamase enzymes which are produced by several clinically important microorganisms. The ability of these microorganisms to produce β-lactamase activities is a major factor in the spread of antibiotic resistance among clinically relevent microorganisms.

Clavulanic acid, which also comprises a β-lactam ring, is a potent inhibitor of β-lactamases and has been used successfully in combination with β-lactam antibiotics in the treatment of infections caused by β-lactamase producing microorganisms. Clavulanic acid is Z-(2R,5R)-3-(β–hydroxy- ethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0] heptane-2-carboxylic acid.

An understanding of the biosynthesis of these β-lactam molecules is central to improvements in the manufacture of existing antibiotics, for example Augmentin (Trade Mark of SmithKline Beecham plc), and for designing novel antibacterial compounds. Although the biosynthetic pathways of penicillins and cephalosporins are well studied, much remains to be learned about the biosynthesis of clavulanic acid.

Several steps in the clavulanic acid biosynthetic pathway have been elucidated, predominantly those within the central part of the pathway, and in some cases their corresponding enzymatic activities have been identified. These enzymatic activities have been the subject of several publications and patent applications, for example EP 0349121 concerns clavaminate acid synthase, WO 95/03416 discloses the enzyme clavulanic acid dehydrogenase and WO 94/12654 the proclavaminate amidinohydrolase.

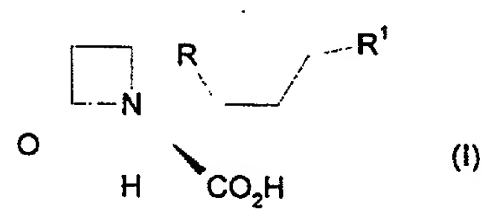
It has been shown that in *Streptomyces clavuligerus*, a clavulanic acid producing organism, the genes responsible for the biosynthesis of clavulanic acid are clustered

together close to the gene cluster involved in the biosynthesis of the penicillin and cephalosporin molecules which are also produced by this organism (Jensen, SE et al, 1993, Industrial Microorganisms, Basic and Applied Molecular Genetics, p169-176; American Society for Microbiology). The DNA sequence and predicted open reading frames for the clavulanic acid biosynthesis cluster are disclosed in Canadian patent application no. 2108113. This patent application discloses an 11.6kb fragment which encompasses 8 open reading frames (orf2 to orf9), which, when introduced into a non-clavulanic acid producing *Streptomyces* strain, confers the ability to produce clavulanic acid. The functions of some of the proteins predicted to be encoded by these orfs have been deduced based on known activity (eg. orf 5 is known to encode clavaminate synthase II; Marsh et al, 1992, Biochem, 31 p12648-12657) or by similarity with known proteins (eg. orf2 shows a high level of homology with acetohydroxyacid synthase (Canadian patent application no. 2108113)). Some proteins, however, remain of unknown function.

The present invention is based on the finding that the orf3 gene encodes an enzyme that is alone capable of the conversion of the β-amino acid (formed from arginine and pyruvate) into the β-lactam form, early in the clavulanic acid biosynthesis pathway.

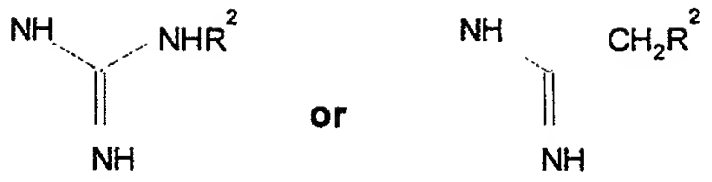
The polynucleotide sequence of orf3 is given in SEQ ID NO:1 and the polypeptide encoded by orf3 is given in SEQ ID NO:2; both sequences are disclosed in Canadian patent application no. 2108113.

Accordingly the present invention provides a process for preparing a β-lactam compound of formula (I) or salt thereof



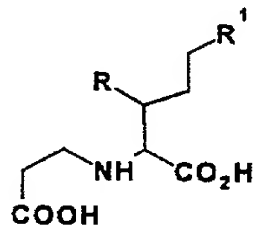
(I)

wherein R is H or OH and R^1 is



and where R^2 = H or C_{1-6} alkyl

by contacting a β-amino acid compound of formula (II) or salt thereof



(II)

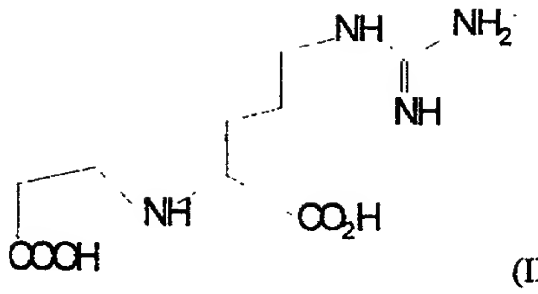
where the variables are as defined in formula (I)

with a polypeptide having at least 95% identity to the amino acid sequence of SEQ ID NO:2, over the entire length of SEQ ID NO:2, and having β-lactam synthetase activity.

Compounds of formula (I) can be converted thereafter to clavulanic acid or other clavam compounds by conventional means. By 'other clavams' we mean compounds containing a 7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane nucleus [J.C.S., Chem. Commun. (1979), 282].

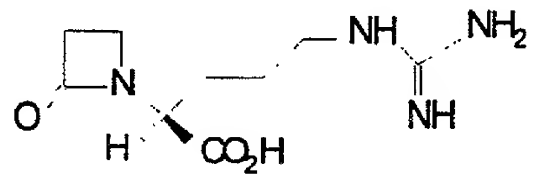
In a further aspect the compound of formula (I), prepared in accordance with the process of the invention, is converted to other clavams and clavam derivatives by treatment with an enzyme system derived from *Streptomyces clavuligerus*.

In a preferred embodiment the process concerns contacting a β-amino acid compound of formula (III) with said polypeptide having β-lactam synthetase activity. The compound of formula (III) is N^2-(2-carboxyethyl)-(S)-arginine first disclosed in WO 94/12654. Methods for the production of a compound of formula (III) are disclosed in WO 94/12654.



(III)

Contacting a compound of formula (III) with a polypeptide having β-lactam synthetase enzyme results in the closure of the β-lactam ring to give a β-lactam compound of formula (IV), which is (2S)-5-guanidino-2-(2-oxo-azetidin-1-yl)pentanoic acid.



(IV)

In a preferred aspect the compound of formula (IV), prepared in accordance with the process of the invention, is converted to clavulanic acid by treatment with an enzyme system derived from *Streptomyces clavuligerus* . The enzyme system includes the enzymes of the clavulanic acid biosynthetic pathway, for example clavaminic acid synthase (Baldwin, JE et al, J.Chem.Soc.,Chem.Commun. 500); proclavaminate amidinohydrolase (disclosed in WO 94/12654); clavulanic acid dehydrogenase (disclosed in WO 95/03416) or any one, or combinations of, the enzymes encoded by the clavulanic acid biosynthesis gene cluster disclosed in Canadian patent application no. 2108113.

Such enzymes may be provided in isolated form, in recombinant hosts transformed with cloned genes encoding the enzymes or by contact with *Streptomyces clavuligerus* whole cells.

The polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2 will herein be referred to as β-lactam synthetase. The β-lactam synthetase preferably has 97-99% identity with the amino acid sequence of SEQ ID NO:2. Such polypeptides include those comprising the amino acid sequence of SEQ ID NO:2. In a most preferred aspect the polypeptide has the amino acid sequence of SEQ ID NO:2.

Polypeptides useful in the present invention will possess the β-lactam synthetase activity. In one aspect, modified polypeptides may be employed which increase the titres of compounds of formula (I) or (IV) or downstream products.

In a further preferred embodiment the process of the invention includes contacting the compound of formula (II) or formula (III) with a β-lactam synthetase enzyme purified from the natural source, for example from *Streptomyces clavuligerus* in a cell-free environment. A process according to the invention for preparing an enzyme having β-lactam synthetase activity comprises the steps:

a) culturing *Streptomyces clavuligerus*,

b) harvesting and lysing the mycelium, and

c) isolating a polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2 and having β-lactam synthetase activity.

A cell-free extract is produced, preferably by sonication or other disruption of the microorganisms, optionally thereafter removing cell debris, leaving the β-lactam synthetase enzyme in solution. This solution is then fractionated to isolate the β-lactam synthetase enzyme. The enzyme may be prepared by culturing the microorganism in a conventional manner, especially under aerobic conditions in a suitable liquid or semi-solid medium. In general, carbon and nitrogen sources which microorganisms can assimilate and inorganic salt nutrients essential for the growth of the microorganisms are included in the culture medium.

The culture medium should contain a source of metal ions such as, for example, iron. The culture conditions may be a temperature in the range of from 10°C to 80°C and pH in the range of from 3 to 10. Preferred conditions are from 20°C to 30°C at a pH of from 5 to 9, suitably, for example, about pH 7, for 0.5 to 5 days.

The enzyme may be isolated and used in purified form, partially purified form, as obtained in an impure state, as a filtrate from a disrupted cell preparation, or as a crude cell homogenate. The enzyme can be recovered and purified from the disrupted cell preparation by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high

individual stereoisomers by conventional means, for example by the use of an optically active salt as a resolving agent or by stereoselective removal of a protecting group using a suitable enzyme, for example an esterase such as subtilisin. In mixtures of diastereoisomers of the compounds the ratio of diastereoisomers may be changed by treatment with a non-nucleophilic base, for example 1,5-diazabicyclo[4.3.0]non-5-ene.

Suitable optically active compounds which may be used as resolving agents are described in 'Topics in Stereochemistry', Vol. 6, Wiley Interscience, 1971, Allinger, N.L. and Eliel, W.L., Eds.

Alternatively, any enantiomer of a compound of formula (I) may be obtained by stereospecific synthesis using optically pure starting materials of formula (II) of known configuration.

Instead of employing a cell-free system, the process of this invention may also be operated using an intact host microorganism expressing the β-lactam synthetase enzyme through recombinant means, under conditions enabling conversion of the compound of formula (II) to the β-lactam compound of formula (I). The precursor compound of formula (II) or (III), or salt thereof, is provided and contacted with the microorganism to produce the compound of formula (I) or (IV) respectively, or salt thereof. The microorganism may be in the form of a growing culture, resting culture, washed mycelium, immobilised cells, or protoplasts.

In a further embodiment a cell-free system, derived from the recombinant organism, may be used to carry out the process of the invention. The cell-free extract may be prepared, and the enzyme purified, as hereinbefore described.

The β-lactam synthetase polypeptides useful in the present invention may be prepared by the use of β-lactam synthetase polynucleotides. Such polynucleotides include isolated polynucleotides comprising a nucleotide sequence encoding a polypeptide which has at least 95% identity to the amino acid sequence of SEQ ID NO:2, over the entire length of SEQ ID NO:2. In this regard, polypeptides which have 97-99% identity are highly preferred. Such polynucleotides include a polynucleotide comprising the nucleotide sequence contained in SEQ ID NO:1 encoding the polypeptide of SEQ ID NO:2.

The invention also provides the use of polynucleotides which are complementary to the above described polynucleotides.

Polynucleotides of the present invention may be obtained, using standard cloning and screening techniques from natural sources such as genomic DNA libraries, PCR from genomic DNA or can be synthesized using well known and commercially available techniques. Certain polynucleotides of the invention and the β-lactam synthetase polypeptides encoded by them are obtainable from *Streptomyces* species. In a preferred aspect the polynucleotides and polypeptides of the invention are obtainable from *Streptomyces clavuligerus*.

The polynucleotides used in the present invention may be DNA or alternatively RNA.

Recombinant polypeptides of the present invention may be prepared by processes well known in the art from recombinant host cells comprising recombinant expression vectors. A process of the invention for preparing an enzyme having β-lactam synthetase activity comprises the steps:

a) culturing a host microorganism transformed with a recombinant vector comprising a polynucleotide capable of producing the polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2, and having β-lactam synthetase activity, and

b) isolating the polypeptide as defined in (a).

In a further aspect, the present invention provides a recombinant vector comprising a polynucleotide capable of producing a β-lactam synthetase polypeptide when said recombinant vector is present in a compatable host. Compatable hosts include, but are not limited to, *E.coli* and *Streptomyces* species.

Cell-free translation systems can also be employed to produce such proteins using RNA polynucleotides.

Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, such as Davis et al, Basic Methods in Molecular Biology (1986) and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

A great variety of expression vectors can be used, for instance, chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids or from bacteriophages. The expression vectors may contain control regions that regulate as well as engender expression. Generally, any vector which is able to maintain, propagate or express

a polynucleotide to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression vector by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (supra). Appropriate secretion signals may be incorporated into the desired polypeptide to allow secretion of the translated protein into the periplasmic space or the extracellular environment. These signals may be endogenous to the polypeptide or they may be heterologous signals.

In a further aspect the host cell contains a high copy number of the β-lactam synthetase polynucleotide.

Culture of the recombinant host microorganism and isolation of the polypeptide are as described above.

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"β-lactam synthetase activity or β-lactam synthetase polypeptide activity" or "biological activity of the β-lactam synthetase or β-lactam synthetase polypeptide" refers to the enzyme activity which is capable of catalysing the conversion of compound (II) to (I), and in a preferred embodiment the conversion of compound (III) to (IV).

"Isolated" means altered "by the hand of man" from the natural state. If an "isolated" composition or substance occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one

or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications.

"% identity", as known in the art, is a measure of the relationship between two polypeptide sequences or two polynucleotide sequences, as determined by comparing their sequences. In general, the two sequences to be compared are aligned to give a maximum correlation between the sequences. The alignment of the two sequences is examined and the number of positions giving an exact amino acid or nucleotide correspondence between the two sequences determined, divided by the total length of the alignment and multiplied by 100 to give a % identity figure. This % identity figure may be determined over the whole length of the sequences to be compared, which is particularly suitable for sequences of the same or very similar length and which are highly homologous, or over shorter defined lengths, which is more suitable for sequences of unequal length or which have a lower level of homology.

Methods for comparing the identity of two or more sequences are well known in the art. Thus for instance, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux J et al, Nucleic Acids Res, 12, 387-395, 1984, available from Genetics Computer Group, Madison, Wisconsin, USA), for example the programs BESTFIT and GAP, may be used to determine the % identity between two polynucleotides or two polypeptide sequences. BESTFIT uses the "local homology" algorithm of Smith and Waterman (Advances in Applied Mathematics, 2, 482-489, 1981) and finds the best single region of similarity between two sequences. BESTFIT is more suited to comparing two polynucleotide or two polypeptide sequences which are dissimilar in length, the program assuming that the shorter sequence represents a portion of the longer. In comparison, GAP aligns two sequences, finding a "maximum similarity", according to the algorithm of Neddleman and Wunsch (J Mol Biol, 48, 443-453, 1970). GAP is more suited to comparing sequences which are approximately the same length and an alignment is expected over the entire length. Preferably, the parameters "Gap Weight" and "Length Weight" used in each program are 50 and 3, for polynucleotide sequences and 12 and 4 for polypeptide sequences, respectively. Preferably, % identities are determined when the two sequences being compared are optimally aligned.

Other programs for determining identity between sequences are also known in the art, for instance the BLAST family of programs (Altschul S F et al, J Mol Biol, 215, 403-410, 1990, Altschul S F et al, Nucleic Acids Res., 25:389-3402, 1997, available from the National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA and accessible through the home page of the NCBI at www.ncbi.nlm.nih.gov) and FASTA (Pearson W R and Lipman D J, Proc Nat Acad Sci USA, 85, 2444-2448,1988, available as part of the Wisconsin Sequence Analysis Package). Preferably, the BLOSUM62 amino acid substitution matrix (Henikoff S and Henikoff J G, Proc. Nat. Acad Sci. USA, 89, 10915-10919, 1992) is used in polypeptide sequence comparisons including where nucleotide sequences are first translated into amino acid sequences before comparison.

Preferably, the program BESTFIT is used to determine the % identity of a query polynucleotide or a polypeptide sequence with respect to a polynucleotide or a

polypeptide sequence of the present invention, the query and the reference sequence being optimally aligned and the parameters of the program set at the default value.

Examples

All general procedures and recipes for buffers and media were carried out as described in "Molecular Cloning:- A Laboratory Manual", Sambrook, Fritsch and Maniatis, 2nd edition, Cold Spring Harbour laboratory Press (1989).

1) Construction of the vector pBLS1

1.1 Preparation of digested vector:

DNA of the plasmid vector pYZ4 (Zhang & Broome-Smith Gene 1996 p51-57, 1990) was prepared from an *E.coli* culture using standard plasmid miniprep techniques. To 9µl of this miniprep sample was added 1.5µl of EcoRI, 1.5µl KpnI, 1.5µl x10 reaction buffer supplied by the manufacturer and 1.5µl H_2O and the sample digested for 2 hours at 37°C. The enzymes were extracted with phenol/chloroform and the DNA isolated by ethanol precipitation. The DNA pellet was resuspended in 4µl TE.

1.2. Preparation of the 4.1kb EcoRI/KpnI DNA fragment containing orf3:

DNA of a cosmid containing the 11.6kb EcoR1 fragment described in Canadian patent application no. 2108113 was prepared from an *E.coli* culture using standard miniprep techniques. To 9µl of this miniprep sample was added 1.5µl of EcoRI, 1.5µl KpnI, 1.5µl x10 reaction buffer and 1.5µl H_2O and the sample digested for 2 hours at 37°C. The enzymes were extracted with phenol/chloroform and the DNA isolated by ethanol precipitation. The pellet was resuspended in 4µl TE.

1.3. Ligation:

The 4µl sample of the 4.1kb DNA fragment from example (1.2). was mixed with 4µl of digested vector from example (1.1). To this was added 1µl of x10 ligase buffer and 1µl of T4 ligase. Following incubation overnight at 4°C, 2.5µl of the solution was used to transform 80µl of *E.coli* XL1-Blue competent cells (Stratagene), plated onto LB agar plates (+ kanamycin) and incubated overnight at 37°C. Miniprep DNA isolation and subsequent restriction digest analysis (BamHI/BglII, HindIII/SacI, HindIII/SphI, SalI,

BamHI/NotI) of several of the kanamycin resistant transformants confirmed that a plasmid had been obtained containing the 4.1 kb EcoRI/KpnI fragment containing orfs2 and 3 of the clavulanic acid gene cluster. This plasmid was named pBLS1.

2) Construction of the vector pBLS2

2.1. Preparation of digested vector:

A 1.5µl sample of the pET-24d(+) expression vector (Novagen) was diluted with 9µl with H_2O. To this was added 1.5µl of BamHI, 1.5µl of x10 restriction buffer and 3.0µl H_2O and the sample was incubated for 2 hours at 37°C. The restricted vector was then dephosphorylated by treating with 1U calf alkaline intestinal phosphatase (New England Biolabs), at 37°C for 1 hr. The mixture was analysed by agarose gel and a DNA fragment of 5.4Kb was isolated, finally eluting into 20µl H_2O.

2.2. Preparation of the insert fragment:

A 50µl sample of pBLS1 vector DNA from example (1.3) was isolated from the *dam* *E.coli* strain GM2163 (New England Biolabs). The use of this strain was essential to facilitate cleavage of the DNA by the restriction enzyme BclI. To 9µl of this was added 1.5µl BclI, 1.5µl of x10 BclI buffer, and 3.0µl H_2O before incubating at 37°C for 1 hr. The mixture was analysed by agarose gel and a DNA fragment of 2 kb isolated and eluted in 10ml H_2O.

2.3. Ligation:

A 6.5µl sample of DNA prepared from example (2.2) was mixed with 1µl of DNA prepared from example (2.1). To this were added 1µl of x10 ligase buffer, 0.5µl ATP (1mM) and 1 µl of T4 ligase. The mixture was incubated at 16°C, for 16 hours and 2.5 µl of the solution was used to transform 40µl of XL1-Blue competent cells (Stratagene) before being plated onto LB agar plates (+ kanamycin) and incubated overnight. Ten kanamycin resistant colonies were picked at random, grown up and the plasmid DNA isolated by miniprep. Restriction digest with SalI indicated that one clone contained the 2 kb BclI DNA fragment as required. This clone was analysed by further restriction digests (SmaI, Bgl II, NcoI and SalI) which generated the expected fragmentation patterns confirming that orf 3 had been cloned. This plasmid was named pBLS2.

3) PCR of orf3 fragment from pBLS2

PCR amplification of the targeted gene was carried out using standard PCR techniques. pBLS2 miniprep DNA from example (2.3) was used as template DNA. Primers were used at a concentration of 10μM in TE. The 10x ThermoPol reaction buffer used contained 200mM Tris-Cl (pH 8.8), 20 mM $MgSO_4$, 100mM KCl, 100mM $(NH_4)_2SO_4$, 1% Triton X-100. dNTP solutions were used at a concentration of 100nM.

Because the pET24a expression vector was used, the 5' primer incorporated an Nde1 site in frame with the start codon and the 3' primer a BamHI site as shown below:

5' primer:

Nde1

5' G GAA TCC *CAT* *ATG* GGG GCA CCG GTT CTT C 3'

3' primer:

BamH I

5' CGC *GGA* *TTC* CTA GGC CGC CCC CCG CG 3'

The underlined portions of the primer sequences denotes coding region.

The following reaction components were all added and the mixture heated to 96°C for 4 minutes before the DNA polymerase was added. The reaction mixture was overlaid with 100μl mineral oil before the reaction was continued.

The optimised reaction mixture contained the following:

H_2O	50.5 μl
DMSO (50%)	20 μl
Buffer	10 μl
$MgSO_4$	2.5 μl
dNTPs	4 x 1 μl
5' Primer	1 μl

3' Primer	1 µl
DNA Template	10 µl
Vent DNA polymerase (New England Biolabs)	1 µl.

Temperature profile:

1. 96°C 4 min

then add enzyme and mineral oil

2. 95°C 4 min
3. 60°C, 1.5 min
4. 72°C 2 min
5. 95°C 1 min
6. 60°C, 1.5 min
7. 72°C 2 min
8. 25 cycles of steps 5 to 7
9. 95°C 1 min
10. 60°C 1.5min
11. 72°C 10 min
12. 4°C incubate

The expected PCR product (1.5kb) was isolated from agarose gel and eluted into 30µl TE.

4) Construction of the vector pBLS3.

4.1. Preparation of digested vector:

A 45µl sample of the pET-24a(+) expression vector (Novagen) isolated by DNA miniprep was digested using 4µl BamHI, 4µl NdeI and 6µl of x10 reaction buffer. The mixture was incubated at 37°C for 18 hrs and 5µl was analysed by agarose gel electrophoresis. The DNA fragment at 5.4kb was isolated and eluted into 5µl of TE.

4.2. Preparation of the insert fragment:

30µl of the orf3 PCR product from example (3) were digested with 3µl BamHI, 3µl NdeI, with 4µl of x10 reaction buffer. The mixture was incubated at 37°C for 18 hrs and

all of the mixture analysed by agarose gel electrophoresis. A DNA fragment corresponding to a size of 1.5kb was isolated, and eluted in 5µl H_2O.

4.3. Ligation:

A 1.0µl sample of DNA from example (4.2) was mixed with 3.0 µl of DNA from example (4.1). To this was added 1µl of x10 ligase buffer, 1.0µl ATP (1mM), 1.0µl of T4 ligase and 3.0µl of H_2O. The mixture was incubated at 23°C for 2 hours then at 16°C, for 16 hours. The mixture (5µl) was transformed into 100µl XL1-Blue before being plated onto LB agar plates (+ kanamycin) and incubated overnight at 37°C. Miniprep DNA isolation and subsequent restriction digest analysis of several of the kanamycin resistant transformants confirmed that the expected plasmid had been obtained containing the 1.5kb PCR generated fragment containing orf3. As further confirmation, the DNA sequence of the cloned PCR product was confirmed. This plasmid was named pBLS3.

5) Isolation of Protein from *E.coli* fermentations

A 50µl sample of pBLS3 vector DNA from example (4) was isolated from a 1.5ml culture of XL1- Blue(pBLS3). To 100µl of *E.coli* BL21(DE3) competent cells (New England Biolabs) were added 5µl of this mini prep DNA. The cells were then used to inoculate LB agar plate (+ kanamycin) and the plates were incubated overnight at 37°C.

5.1. Small scale

A single colony of *E.coli* BL21(DE3) containing pBLS3 was used to inoculate 5ml of LB containing Kanamycin. This was grown at 37°C for 4 hours, then 2ml were used to inoculate 100mls of 2YT (+ kanamycin) and left to grow for another 2 hours at 37°C. The cultures were then induced with IPTG to a level of 0.1mM, and left to grow at 37°C overnight (17 hours) before being harvested by centrifugation at 4°C and stored at -20°C.

5.2. Large Scale

Larger scale fermentation was achieved by using a single colony of *E.coli* BL21(DE3) containing pBLS3 to inoculate 200ml 2YT which was grown overnight at 37°C. 10ml of

this was used to inoculate 500ml of 2YT and grown for 5 hours before being induced to a level of 0.1mM IPTG and left to grow for a further 4 hours before being harvested as before.

6) Purification of the orf3 protein

6.1 Sample preparation

The volumes of the buffers used were scaled according to the weight of cells to be processed. Typically 30g of cells (prepared as described in example (5)) were resuspended in 90ml Lysis buffer; 50mM Tris-HCl (pH8), 100mM NaCl, 10% glycerol, 2mM DTT, 3mM EDTA. Then lysozyme was added to produce a final concentration of 0.5mg/ml and the mixture stirred for 20 minutes at 4°C. The cells were then lysed using a sonicator (5 x 10 seconds). 15mM $MgCl_2$ and 20µg/ml DNase1 were then added and the mixture stirred for another 15 minutes at 4°C. The mixture was then centrifuged for 30 minutes at 20,000rpm at 4°C. The supernatant was removed and 0.05% polyethylimine and 1% streptomycin added. The mixture was then stirred for 20 minutes at 4°C to precipitate out the DNA which was removed by centrifugation at 20,000rpm at 4°C for 25 minutes. The supernatant was filtered before being mixed with 150ml of buffer (buffer A for FPLC described in example 6.2) ready for loading onto the FPLC system.

6.2 FPLC Set-up

FPLC was carried out on a Pharmacia LCC-500 plus gradient controller with a P-500 pump and Pharmacia FRAC-200 rotary fraction collector. Elution of proteins was monitored by U.V. absorption at 280nm.

ColumnQ Sepharose, 100ml

Buffer A	25mM Tris-HCl(pH 8.), 2mM DTT, 1mM EDTA
Buffer B	25mM Tris-HCl(pH8.0), 2mM DTT, 1mM EDTA, 2M NaCl
Gradient	0 - 8% B over 50ml at 8ml/min
	8 - 18% B over 500ml at 8ml/min
Fractions	10ml fractions collected

Using this system 50 x 10ml fractions were collected. Analysis by SDS-PAGE gel indicated that the fractions containing orf 3 were 13 to 23. This was confirmed by further analysis of these extracts by Western blot analysis using an anti-orf3 polyclonal antibody

raised against a 16 amino acid peptide corresponding to a region close to the C-terminus of the predicted orf3 protein (VGGGRHPSEVDTDDVC) (Canadian patent application no. 2108113) which gave a positive result for the 56 KDa protein corresponding to the predicted size of the orf3 protein.

From SDS-PAGE gel anaylsis of the protein fractions collected by this method it was estimated that the best fractions containing orf3 were ca. 40% pure.

As a final confirmation of the orf3 protein N-terminal amino acid sequencing was undertaken using standard Edman degradation methodology. The sequence generated from this showed 100% sequence identity with the predicted sequence (Figure 12 in Canadian patent application no. 2108113), ie.

NH_2-GAPVLPAAFGFLASARTGGG

7) Assays for functionality of orf3 protein

The crude extracts prepared from the recombinant *E. coli* containing the pBLS3vector (example 5) and the purified protein product (example 6) were tested for the ability to catalyse the conversion of the compound (III) to compound (IV). Both compound (III) and compound (IV) were prepared synthetically following previously published methods (Baldwin et al J.Chem Soc. Chem Comm. 1993 p500-2 and Elson et al J. Chem Soc. Chem Comm. 1993 p1212-4).

7.1

Protein extracts were assayed for the conversion of compound (III) to compound (IV) under the following conditions:

Assay mix	Enzyme* (ca.3mg/mL)	150ml
	Compound (III)(1M)	10ml
	MOPS (1M, pH7.4)	20ml
	$Mg(AcO)_2$ (1M)	5ml
	ATP (0.5M)	5ml

* negative controls were: 150ml extract fractions containing no orf3 protein or 150ml water.

The above mixture was heated at 37 °C for 15 minutes before being analysed by the HPLC methods described below .

7.2 Underivitised HPLC assay conditions.

This assay was carried out using the following equipment and conditions

Waters 746 data module

Waters 600E system controller

Waters 712 WISP injector

Waters 848 tunable absorbance detector

Column	ODS, 250mm x 1mM
Mobile phase	H_2O
Flow rate	4ml/min
Detection	218nm
Sample injected	200ul

When extracts were used in this assay which had been shown to contain the orf3 protein (as described in example (6)) a peak was seen at 10.94 mins on the HPLC trace which correlated with the retention time for compound (IV). This peak was not detected if compound (III), ATP or the orf3 containing protein extract was omitted from the assay. The peak could also not be detected if other protein extracts, which did not contain the orf3 protein, were collected from the FPLC and assayed by the same method.

The peak identified at 10.94 mins was manually collected and submitted for electrospray mass spectrometry analysis where it was shown to have a molecular weight of 229, which is the positive ion equivalent of compound (IV). For final confirmation that this peak corresponded to compound (IV) several samples of the peak were combined, freeze dried and submitted for 500MHz nmr analysis. The spectrum produced in this test was consistent with the product of the reaction being compound (IV).

7.3 Benzoin derivatised HPLC Assay Conditions

The protocol used was Kai et al. (M. Kai, T. Miyazaki, M. Yamaguchi, Y. Ohkura, J.Chromatog. **268**, 417-424, 1983) which assays for compounds containing guanidino functionalities using a fluorescence detector. The following equipment and conditions were used:

Waters 746 data module

Waters 600E system controller

Waters 712 WISP injector

Waters 848 tunable absorbance detector

Jasco FP-920 intelligent fluorescence detector

Column	Phenyl 250x4.6mm			
Buffer A	Methanol : Water : 0.5M Tris-HCl(pH8.5) 50:35:15			
Buffer B	Methanol : Water : 0.5M Tris-HCl(pH8.5) 80:5:15			
Program	Time	Flow(ml/min)	A	B
	0	0.8.	100	0
	25	0.8	0	100
	34	0.8	0	100
	35	0.8	100	0

Reagents

Benzoin solution (4.0mM)

b-Mercaptoethanol (0.1M)-sodium sulphite (0.2M) solution

Sodium hydroxide (2.0M)

Tris-HCl (0.5M, pH 9.2)- Hydrochloric acid (2M)

Method

To a 200µl sample of the incubated assay mixture described in example (7.1) was added 100µl of benzoin solution and 100µl of β-mercaptoethanol solution. Then whilst on ice 200µl of NaOH was added. The mixture was then heated on a hot block at 100°C for 5 minutes and returned to ice. Whilst on ice 200µl of Tris-HCl solution was added. 200µl of this sample were then injected onto the column.

A portion of the collected fraction from the HPLC purified reaction mixture described in example (7.2) was derivatised with benzoin. This sample showed a retention time that matched with that of compound (IV). The crude reaction mixture was also derivatised with benzoin and this gave peaks correlating to unused compound (III) and the product compound (IV).

As found with the results from the underivitised HPLC experiment from example (7.2), a peak corresponding to compound (IV) was only detected when the orf3 protein was present in the assay and not when compound (III), ATP or the orf3 containing

protein extract was omitted. Also a peak corresponding to compound (IV) could not be detected if other protein extracts which did not contain the orf3 protein were collected from the FPLC were assayed by the same method.

These results demonstrate that the orf3 product catalysis the conversion of compound (III) to compound (IV) .To reflect the fact that this reaction produces a monocyclic β-lactam the enzyme was named β-lactam synthetase 1 (BLS 1).

Abbreviations

TE	Tris EDTA buffer (Sambrook et al *supra*)
YT	Yeast Tryptone broth/ agar (Sambrook et al *supra*)
IPTG	Isopropyl β-D-thiogalactopyranoside
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HPLC	High Performance Liquid Chromatography
FPLC	Fast Protein Liquid Chromatography

Sequence information.

SEQ ID NO:1

ATGGGGGCACCGGTTCTTCCGGCTGCCTTCGGGTTCCTGGCCTCCGCCCGAACGGGCGGGGGCCGGGCCCCCG
GCCCGGTCTTCGCGACCCGGGGCAGCCACACCGACATCGACACGCCCCAGGGGGAGCGCTCGCTCGCGGCGAC
CCTGGTGCACGCCCCCTCGGTCGCGCCCGACCGCGCGGTGGCGCGCTCCCTCACCGGCGCGCCCACCACCGCG
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ACGCGGAGCTGGTCCTGCGGCTGCTGGAACGCTATGACCTGCATGCCTTCCGGCTGGTGAACGGGCGCTTCGC
GACCGTGGTGCGGACCGGGGACCGGGTCCTGCTCGCCACCGACCACGCCGGTTCGGTGCCGCTGTACACCTGT
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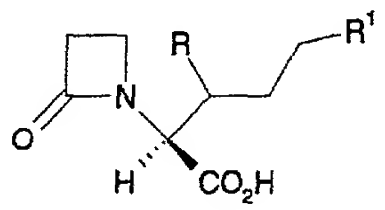
GCTCAAGCGGCGGCACGGCCGGGACAAGTGGGTGCTGCGCGCCGCGATGGCCGACGCCCTCCCGGCGGAGACC
GTCAACCGGCCCAAGCTGGGCGTCCACGAGGGCTCGGGCACCACGTCCTCGTTCTCCCGGCTGCTGCTGGACC
ACGGTGTCGCCGAGGACCGCGTCCACGAGGCGAAGCGGCAGGTGGTGCGCGAGCTGTTCGATCTCACGGTCGG
GGGCGGACGGCACCCCTCCGAGGTGGACACCGACGATGTGGTGCGCTCCGTGGCCGACCGGACCGCGCGGGGG
GCGGCCTAG

SEQ ID NO:2

GAPVLPAAFGFLASARTGGGRAPGPVFATRGSHTDIDTPQGERSLAATLVHAPSVAPDRAVARSLTGAPTTAV
LAGEIYNRDELLSVLPAGPAPEGDAELVLRLLERYDLHAFRLVNGRFATVVRTGDRVLLATDHAGSVPLYTCV
APGEVRASTEAKALAAHRDPKGFPLADARRVAGLTGVYQVPAGAVMDIDLGSGTAVTHRTWTPGLSRRILPEG
EAVAAVRAALEKAVAQRVTPGDTPLVVLSGGIDSSGVAACAHRAAGELDTVSMGTDTSNEFREARAVVDHLRT
RHREITIPTTELLAQLPYAVWASESVDPDIIEYLLPLTALYRALDGPERRILTGYGADIPLGGMHREDRLPAL
DTVLAHDMATFDGLNEMSPVLSTLAGHWTTHPYWDREVLDLLVSLEAGLKRRHGRDKWVLRAAMADALPAETV
NRPKLGVHEGSGTTSSFSRLLLDHGVAEDRVHEAKRQVVRELFDLTVGGGRHPSEVDTDDVVRSVADRTARGA
A

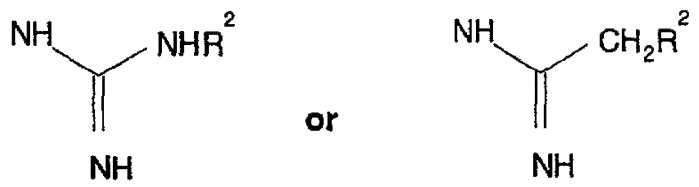
Claims

1. A process for preparing compounds of formula (I)



(I)

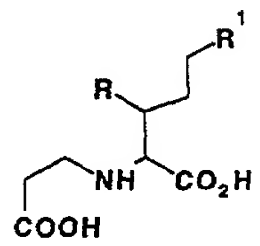
wherein R is H or OH and R^1 is



or

and where R^2 = H or C_{1-6} alkyl

by contacting a compound of formula (II)

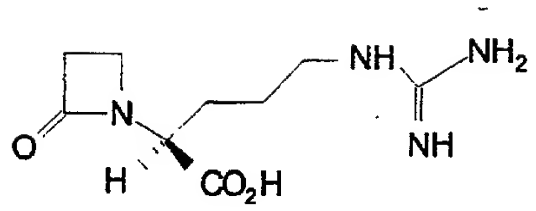


(II)

where the variables are as defined in formula (I)

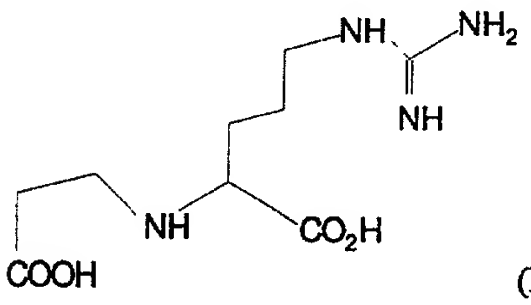
with a polypeptide having at least 95% identity to the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2, and having β-lactam synthetase activity.

2. A process according to claim 1 for preparing a compound of formula (IV)



(IV)

wherein the compound of formula (II) is N^2-(2-carboxyethyl)-(S)-arginine; formula (III)



(III)

3. A process according to claim 1 or 2 wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:2.

4. A process according to claim 1 or 2 wherein the polypeptide has the amino acid sequence of SEQ ID NO:2.

5. A process according to any one of claims 1 to 4 wherein the polypeptide having β-lactam synthetase activity is obtainable from *Streptomyces* species.

6. A process according to claim 5 wherein the *Streptomyces* species is *Streptomyces clavuligerus*.

7. A recombinant vector comprising a polynucleotide capable of producing the polypeptide defined in claim 1 when said vector is present in a compatable host.

8. A recombinant vector according to claim 7 comprising a polynucleotide selected from the group:

a) a polynucleotide encoding a polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2;
b) a polynucleotide comprising the polynucleotide sequence of SEQ ID NO:1; or
c) a polynucleotide having the polynucleotide sequence of SEQ ID NO:1.

9. A process according to any one of claims 1 to 6 wherein the polypeptide is expressed from a recombinant vector according to claim 7 or 8.

10. A host microorganism containing a recombinant vector of claims 7 or 8.

11. A host microorganism according to claim 10 which is selected from *Streptomyces*, or *E.coli*.

12. A process for preparing an enzyme having β-lactam synthetase activity which comprises the steps:
a) culturing *Streptomyces clavuligerus*,
b) harvesting and lysing the mycelium, and
c) isolating a polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2, and having β-lactam synthetase activity.

13. A process for preparing an enzyme having β-lactam synthetase activity which comprises the steps:
a) culturing a host microorganism transformed with a recombinant vector according to claims 7 or 8, and
b) isolating the polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2 over the entire length of SEQ ID NO:2, and having β-lactam synthetase activity.

14. A process for preparing clavulanic acid comprising preparing a compound of formula (IV) in accordance with claim 2 or any claim dependent thereon, and then

converting the compound of formula (IV) to clavulanic acid by treatment with an enzyme system derived from *Streptomyces clavuligerus*.

#8

Docket No.: P32085 PCT/GB99/02301

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PROCESS FOR PREPARING CLAVAM DERIVATIVES BY USING POLYPEPTIDES HAVING BETA-LACTAM SYNTHETASE ACTIVITY

the specification of which (check one)

[ ] is attached hereto.

[X] was filed on 15 July 1999 as Serial No. PCT/GB99/02301
and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or Inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Number	Country	Filing Date	Priority Claimed
9815666.4	Great Britain	17 July 1998	Yes

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

Application Number	Filing Date

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Serial No.	Filing Date	Status

I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

Customer Number 20462.

Address all correspondence and telephone calls to Zoltan Kerekes, SmithKline Beecham Corporation, Corporate Intellectual Property-U.S., UW2220, P.O. Box 1539, King of Prussia, Pennsylvania 19406-0939, whose telephone number is 610-270-5024.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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SEQUENCE LISTING

<110> Barton, Barry
McNaughton, Heather Jane
Schofield, Christopher Joseph
Thirkettle, Jan Edward

<120> Process for Preparing Clavam Derivatives
by Using Polypeptides Having Beta-Lactam Synthetase Activity

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